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Departamento Biologia

Tullus Ullus Bergmann Filho

O USO DE BIOMARCADORES EM PEIXES DE UM ESTUÁRIO POLUÍDO, SP, BRASIL

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica Dr. Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, e da Drª Susana Loureiro, Investigadora Auxiliar do CESAM, Universidade de Aveiro.

´´I´ve seen scientists, who were persecuted, ridiculed, deprived of jobs income, simply because the facts that they discovered led them to the inconvenient truth that they insisted on telling.´´

– AL GORE

o júri

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palavras-chave

Biomarcadores, *Cathorops spixii*, ambiente estuarinos, contaminação ambiental.

Resumo

O ecossistema estuarino de Santos e São Vicente localiza-se no sudeste do Brasil. Estudos preliminares indicam níveis elevados de contaminação causados por efluentes industriais, moradias irregulares (palafitas), poluição difusa, esgoto doméstico e depósitos de lixo. Uma grande quantidade de diferentes compostos químicos resultantes desses impactos têm se acumulado no sedimento ao longo dos anos. Muitos estudos ecotoxicológicos têm sido feitos para investigar os efeitos dos contaminantes, mas poucos estudos utilizando biomarcadores. Nesse estudo, o impacto da contaminação no sedimento, o qual se pode refletir em todo o ecossistema, foi avaliado utilizando o peixe bentônico *Cathorops spixii*. Os biomarcadores utilizados para avaliar os efeitos da contaminação no metabolismo dos peixes foram LPO (peroxidação lipídica), CAT (catalase), GST (glutathione S-transferases) e EROD (ethoxyresorufin-o-deethylas) no fígado. No músculo e cérebro fora analisado a Che (cholinesterase) e no músculo a LDH (lactato desidrogenase). Os resultados indicam diferenças significativas encontradas na fase I e II da detoxificação, provavelmente provocados por elevadas concentrações de PAHs no sedimento e inibições na ChE e LDH devido à presença de pesticidas. Os resultados sugerem que a biotransformação e eliminação de xenobioticos ocorrem na fase I e II da detoxificação e que *C. spixii* demonstrou ser uma espécie eficiente para avaliar os efeitos da poluição no estuário de Santos e São Vicente.

Keywords **Fish biomarkers, *Cathorops spixii*, estuarine environments, environmental contamination**

Abstract

The estuarine ecosystem of Santos and São Vicente is located in south-eastern Brazil and comprehends the biggest marine harbour and one of the biggest industrial areas of Latin America. Preliminarily studies indicated high levels of contamination caused by industrial effluents, wood stacks slams, diffuse wastes, domestic sewage and waste deposits. High levels of different chemical compounds resulting from these impacts have been accumulated in sediments. Several ecotoxicological assays have been carried out to investigate the effects of some local contaminants but only few used biomarkers as effect criteria. In this study, the impact of sediments contamination, which may be reflected to the whole ecosystem, was assessed using the benthonic fish *Cathorops spixii*. The biomarkers used to evaluate the pollution effect on fish metabolism were LPO (lipid peroxidation), CAT (catalase), GST (glutathione S-transferases), and EROD (etho-xyresorufin-o-deethylas) in liver, ChE (cholinesterase) in muscle and brains and LDH (lactate desidrogenase). Results presents significant differences found in phase I and II of detoxification, probably promoted by high concentrations of PAHs in sediment and inhibition of ChE and LDH due to the presence of pesticides. Results suggest that the biotransformation and elimination of xenobiotics occur in the phase I and II of the detoxification process and *C. Spixii* demonstrated to be a good sentinel species to assess pollution effects from Santos and São Vicente estuarine system.

Index

Table of contents

List of Figures

List of Tables

Chapter I – Background, aim, and scope

Estuaries are the most productive marine ecosystems in the world and are essential to the life history and development of many aquatic species, including feeding activities, migration routes, and reproduction. Thus, it is crucial that sediment contamination in estuarine environments and its biological and ecological impairments are fully assessed.

Estuaries receive several anthropogenic inputs from metropolitan areas and industries located around estuaries, and over the last 30 years, estuaries were used as the destination for the cities sewage. Sediment contamination in estuarine environments has been related with effects on benthonic species and also with effects on water column species (Chapman & Wang 2001).

The São Paulo state is located in the southeast of Brazil and the cities of Santos and São Vicente comprehends the main cities of São Paulo coast (fig. 1), one of the most critical regions of water and sediment contamination as results from multiple contamination sources. The Santos and São Vicente region has an area of approximately 794 km^2 (23°30'- 24°S and 46°5'- 46°30'W) (Abessa 2002, Lamparelli et al. 2001, Nishigima et al. 2001).

Figure 1. Estuarine system of Santos and São Vicente, SP, Brazil (from Ecomanage Project, 2008).

As observed in other estuarine environments, contaminants are accumulating in the sediments of Santos estuary which may represent not only a

sink but also a source of contamination to benthic organisms and the water column (Sousa et al. 2007).

The anthropogenic activities in Santos and São Vicente estuarine system include mainly the discharge of domestic and industrial effluents into its rivers and estuaries. The Santos and São Vicente estuarine system comprises multiple contamination sources mainly from the Cubatão industrial pole at the bottom of the estuary (fig. 2), the biggest Latin American Port at Santos channel, sewage outfalls (fig. 3), storm water drains, irregular human occupation (fig. 4) and illegal landfills and dumping sites. In the portuary area, intense loading and dumping activities involving shipping operations, generates 97% of waste which is partly discharged into the sea (Gasparro et al. 2008, Gianesella et al. 2008, Lamparelli et al. 2006, Lamparelli et al. 2005, Lamparelli et al. 2001, Nishigima et al. 2001, Sousa et al. 2007, Sousa et al. 2008).

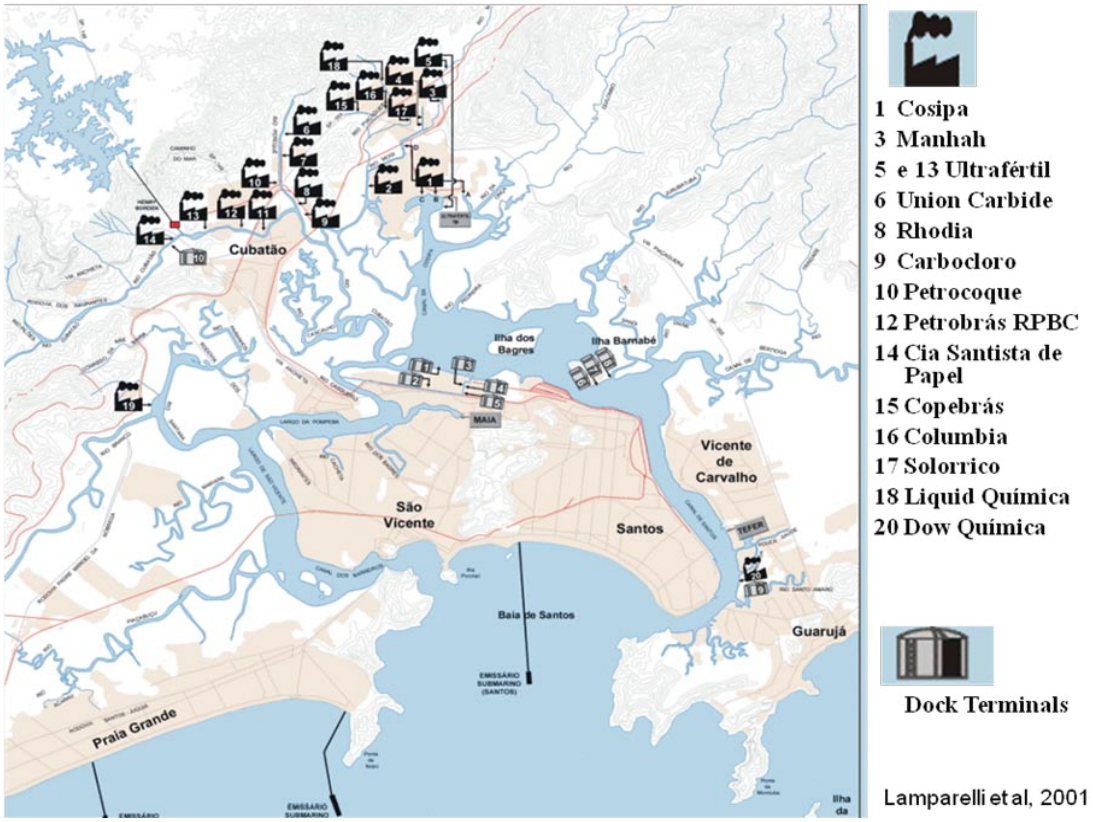

Figure 2. Sources of industrial pollution from Santos and São Vicente estuarine system, SP, Brazil (adapted from Lamparelli et al, 2001).

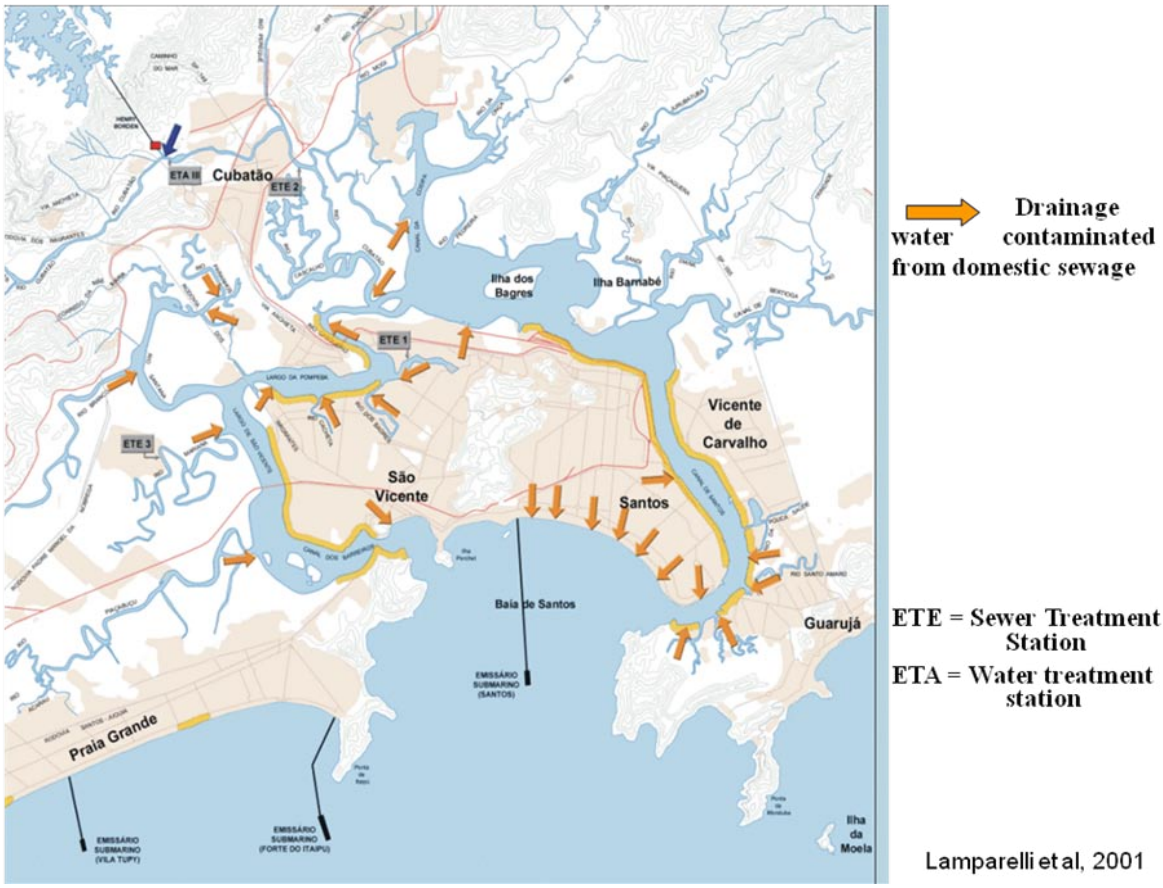

Figure 3. Sources of domestic pollution from drainage waters, sewage treatment station and water treatment station, from Santos and São Vicente estuarine system (adapted from Lamparelli et al, 2001).

Figure 4. Irregular human occupation in estuary margins of Santos and São Vicente estuarine system, SP, Brazil (from Ecomanage Project, 2008).

Santos harbour responds for 30% of the total national exportation and 60% of the total containers movement in the country. Nowadays the annual charge movement is approximately 60 million tons of a variety of products. Santos harbour possesses 12 km of piers on both sides of the Santos channel. However, it is still growing by the acquisition of new lands in the neighbouring areas (Gasparro et al. 2008).

Several contaminants, such as metals (fig. 5), aliphatic and polyaromatic hydrocarbons (PAHs) (fig. 6), had already been detected in potentially toxic concentrations in this area (Gasparro et al. 2008, Lamparelli et al. 2001, Nishigima et al. 2001, Sousa et al. 2007).

The PAH are formed by the incomplete combustion of gasoline, diesel oil and other petroleum products and are carried out into the ocean by atmospheric deposition and rivers. PAHs are associated with particulate and dissolved material and tend to be deposited in the sediments and their presence can be used as indicator of pollution from oil contamination (Nishigima et al. 2001).

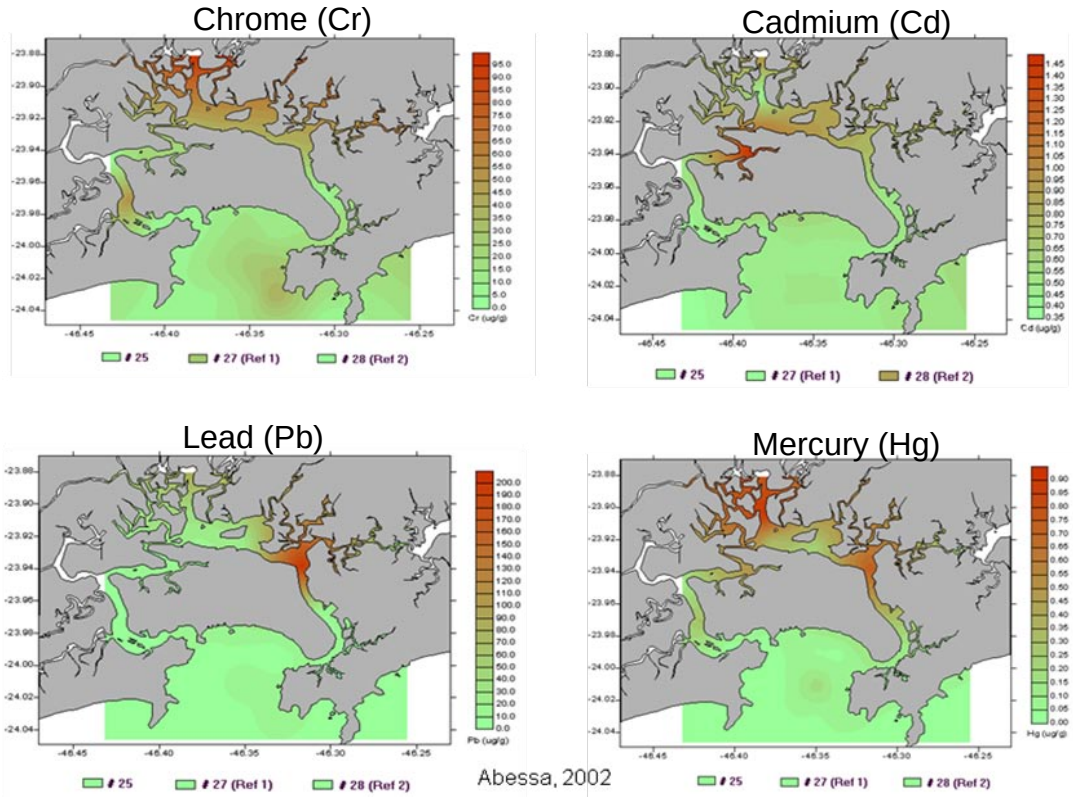

Figure 5. Examples of metals concentrations in sediments from Santos and São Vicente estuarine system, SP, Brazil (from Abessa, 2002).

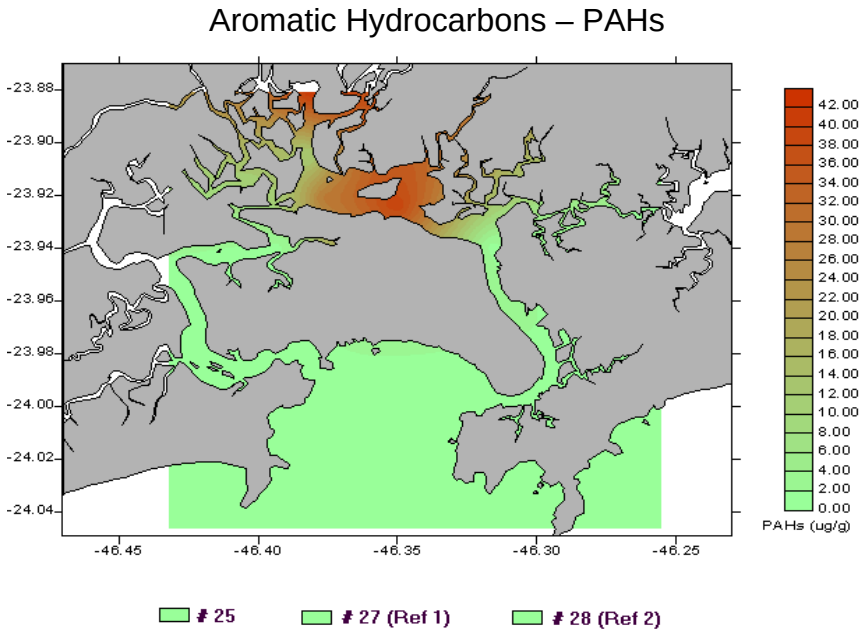

Figure 6. Concentrations of total Polycyclic Aromatic Hydrocarbons (PAHs) in Santos and São Vicente estuarine system, SP, Brazil (from Abessa, 2002).

The estuary is used as food source for about 46% of the region families and the fishing activity represents an important economic activity to the population with minor economic power. Considering this, the estuary contamination is critical when evaluating contaminant contents in fish, crabs and shellfishes. Concentrations of Cu, Ni, Zn, PAHs, dioxins and furan are found in fish above the limit for human consumption in Brazil, indicating the existence of risk to the population health, mainly for fishing collectors and their families (Lamparelli et al. 2007, Lamparelli et al. 2001).

The presence of contamination in Santos and São Vicente estuary is known as an important environmental problem, but the bioavailability and the inherent risk to human health have been less studied. Studies that provide more information about the effects of contamination to organisms of that region are required to help establish programs for pollutants' removal and environmental recuperation of that region (Abessa 2002).

The negative effects of xenobiotic to a population are difficult to identify or conclude through ecotoxicological assay because of the time needed to detect effects, but mainly due to the difficulty to make a bridge between environment and human health. Biomarkers make possible the detection of metabolic alterations

before affecting the organism population and their wide use in fish has been carried out to evaluate the quality of aquatic ecosystems (Oost et al. 2003).

Despite the high mobility, fishes are considered the best organisms to monitor the pollution at aquatic ecosystem (Oost et al. 2003). In this work, the fish species used was the catfish *Cathorops spixii* (fig. 7). This species inhabits the benthonic zone and feed from organism on the sediment. Bottom catfish, as *C. spixii*, are often used in monitoring programs at estuarine environments. Several studies have confirmed the induction of hepatic enzymatic alterations in laboratory exposures (Katsumiti et al. 2008, Murphy & Gooch 1997). Their preference for muddy bottoms, where lipophilic organic contaminants may be higher, their dietary profiles, and abundance in urban estuaries, make catfish species ideal for monitoring organic chemical pollutants and environmental bioavailability (Figueiredo & Menezes 1978, Murphy & Gooch 1997).

Figure 7. Bottom catfish Cathorops spixii (from www.fishbase.org 2008)

Their life cycle includes a permanent contact with potentially contaminated sediments and can lead to the bioaccumulation of chemical substances and the transference of these substances through the trophic chain reaching humans.

The aim of this study was to evaluate the potential toxicity of sediments from Santos and São Vicente estuarine system using biomarkers in bottom catfish species presents in the region and comparing results from biomarkers analysis with chemical analysis made in sediments by annual environmental reports.

The reference site used as reference in this work was the Cananéia estuarine complex. That region is located on the southern coast of São Paulo

State (between latitude 24°40´00´´ and 25°05´00´´S and longitudes 47°25´00´´ and 48°00´00´´W) (fig. 8). The Cananéia estuary was the region of São Paulo state with the biggest number of environmental conservation units, sustainable use units and specially protected areas. The region is also part of the Biosphere Reserve (UNESCO) and had a large permanent preserved Atlantic Forest and mangrove area (Lamparelli et al. 2007).

Figure 8. Cananéia estuarine complex, SP, Brazil (from Ecomanage Project, 2008).

Chapter II – The use of fish biomarkers as indicators of contamination in Santos and São Vicente estuarine system, SP, Brazil.

Introduction

Aquatic pollution is increasing in Brazilian coasts mainly due to the increase of domestic effluent discharges and the presence and intense activities of ports, industries, emissaries, mining activities and others (Sousa et al. 2007).

In the south-eastern Brazil, the estuarine system of Santos and São Vicente located in the São Paulo state, is one of the most critical and polluted regions. The contamination of water, sediment and biota are generated by multiple sources of contamination (i.e. toxic waste, industrial effluents, wood stacks slams, diffuse wastes, domestic sewage and waste deposits) mainly from discharges of pollutants from domestic origin and industrial plants. Other factors like the uncontrolled and inadequate ground occupation and diffuse contamination contribute for the critical environmental state of that region (Gasparro et al. 2008, Lamparelli et al. 2001).

The estuary is used by 46% of the region families as food source and the fishing activity represents an important economic activity to the population with minor economic power. The situation is critical when evaluating the contaminant contents in fish, crabs and shellfishes, where concentrations of Cu, Ni, Zn, PAHs, dioxins and furan are found above the limit for human consumption in Brazil, indicating the existence of risk to human health, mainly for people that live from fishing activities, sea food collectors and their families (Lamparelli et al. 2001).

Although the presence of contaminants in the estuary is well known, the ecological meaning, their bioavailability for aquatic organisms and the inherent risks for human health had not been well studied and evaluated. So the development of studies that contribute to the establishment of effective unpolluted programs and environmental recovery of the region are of crucial importance (Abessa 2002).

Several studies have been carried out to evaluate water and sediment toxicities in Santos and São Vicente estuary using ecotoxicological assays and chemical analysis (Abessa 2002, CETESB 1981, 1985, Gasparro et al. 2008,

Gianesella et al. 2008, Lamparelli et al. 2001, Sousa et al. 2007, Sousa et al. 2008, Tommasi 1985). But only the studies from Pereira (2008) used biomarkers to evaluate toxic effects to organism's metabolism.

The deleterious effects by xenobiotics to a population are difficult to identify through ecotoxicological assays because they need time to be expressed. The use of biomarkers makes possible the detection of physiological alterations caused by xenobiotics before they can be phenotypic detected in organisms or in their populations (Oost et al. 2003).

The use of biomarkers in fish have been widely used to determine the quality of aquatic ecosystems because this organisms play an important role in the aquatic food chain by carrying and accumulating energy from lower to higher levels (Oost et al. 2003).

Catfish species are often used in monitoring programs in estuarine environments. Several studies have confirmed the induction of hepatic enzymes in laboratory exposures. Their preference for muddy bottoms, where lipophilic organic contaminants may be higher, their dietary patterns, and abundance in urban estuaries, make catfish species ideal to monitor the presence and bioavailability of organic chemical pollutants (Murphy & Gooch 1997).

In this study, the bottom catfish *Cathorops spixii* was used as monitor species and tissues (i.e. liver, brain, muscle) collected to measure several enzymatic activity alterations with the aim of evaluating pollution effects on fish metabolism sampled from the Santos and São Vicente estuary.

In the liver, enzymatic activities studied belonged to the phase I of detoxification (EROD, thoxyresorufin O-deethylase), the phase II of detoxification (GST, Glutathione S-transferases) and biomarkers of oxidative stress (LPO, Lipid peroxidation; CAT, Catalase) (Al-Arabi & Goksøyr 2002, Farombi et al. 2007, Lawrence et al. 2003, Oost et al. 2003).

Enzymatic activity measured in the liver has proved to be an important tool for the evaluation of exposure of fish to polluted sites mainly to aromatic pollutants (Murphy & Gooch 1997). Polluted environments generate the production of specific enzymatic responses that are able to modify these compounds into more water-soluble and consequently more excretal metabolites. Enzymatic activities

from phase I of detoxification can be evaluated by the induction of cytochrome P4501A isoenzymes (CYPIA). The cyt P450 isoenymes induction can be determined by the ethoxyresorufin 0-deethylase (EROD) activity. Accurate results have been obtained in the channel catfish *Ictalurus punctatus* from estuarine waters with bottom-feeding characteristics (Murphy & Gooch 1997, Rotchell et al. 1999).

Glutathione S-transferases (GSTs) are a phase II detoxification enzyme family that can minify the toxicity in the liver of environmental chemicals. The first catalytic activity of GSTs is the conjugation of electrophilic compounds which facilitate the excretion of endogenous and xenobiotics substances. Environmental chemicals detoxified by GSTs include carcinogens, pesticides, and reactive intermediates (Trute et al. 2007).

The biomarkers used to evaluate the oxidative stress in fish liver in this work were catalase (CAT) and the lipid peroxidation (LPO). CAT are responsible for facilitate the removal of hydrogen peroxide (H_2O_2) which is metabolized to oxygen (O_2) and water (H_2O) and LPO is a consequence of oxidative stress caused by reactions which leads to several deleterious biochemical reactions (Oost et al. 2003).

Acetylcholinesterase (ChE) inhibition in fish brain and muscle is known to be a good biomarker for organophosphates and carbamates exposure and has been widely used in environmental monitoring (Kirby et al. 2000). The function of ChE is to transform acetylcholine into choline and acetic acid. Acetylcholine is a neurotransmitter with the important role of maintaining normal neural and muscular functions. Inhibition of the ChE enzyme will result in an accumulation of acetylcholine promoting a continuous and excessive stimulation of the nerve/muscle fibers which will result in tremors, paralysis and eventual death. In fish, the inhibition of ChE activity can affect respiration, feeding and swimming (Kirby et al. 2000, Lawrence et al. 2003).

The lactate dehydrogenase (LDH) activity measured in fish muscle is an important enzyme used in aquatic toxicology to evaluate alterations in metabolisms as an indicator in vertebrates and invertebrates (Greco et al. 2007).

LDH activity is an indicative enzyme for energy production and anaerobic metabolism.

The aim of this study was to evaluate the effects of pollution of Santos and São Vicente estuary in the catfish *Cathorops spixii* using a battery of biomarkers. Results will be then related to chemical analysis already performed in sediments (i.e. PAHs and metals) and published in the annual monitoring report for the coast of São Paulo state, Brazil, by Lamparelli et al. (2007) from the Company of Environmental Sanitation (CETESB).

Material and Method

Organism

The organism used in this study is the yellow catfish *Cathorops spixii* (Ariidae, Actinopterygii). It is a tropical demersal catfish well distributed along the coast of South America from the Venezuela coast to the Brazilian south-eastern coast. This species feed mainly from invertebrates and small fishes and it is found in brackish estuaries and shallow coastal marine waters, lagoons, and in hyper saline waters (Figueiredo & Menezes 1978, Katsumiti et al. 2008).

The fish meat is considered of good quality (www.fishbase.org, version 07/2008) and they are considered economically important for fishery activities in the Brazilian Southern region (Gomes & Araújo 2004).

The average fish length sampled is of 186mm. The sexual differentiation is not possible by external observation and that characterization occurs only after extracting fish tissue.

Study Areas

Santos and São Vicente Estuarine System

The estuary system of Santos and São Vicente is located at the central part of the São Paulo state coast (23°30' - 24°S and 46°5' - 46°30' W) (Figure 9). This region is considered of great economic importance, due to the presence of the industrial area of Cubatão city at the end of the estuary, the Santos Port, which is the biggest Port of Latin America, its tourist potential and also by the resources from fishing activities and the mangrove that exists in the estuary. One sampling

point was located near the Bagre island (Santos) and the other in the São Vicente channel (Fig. 9).

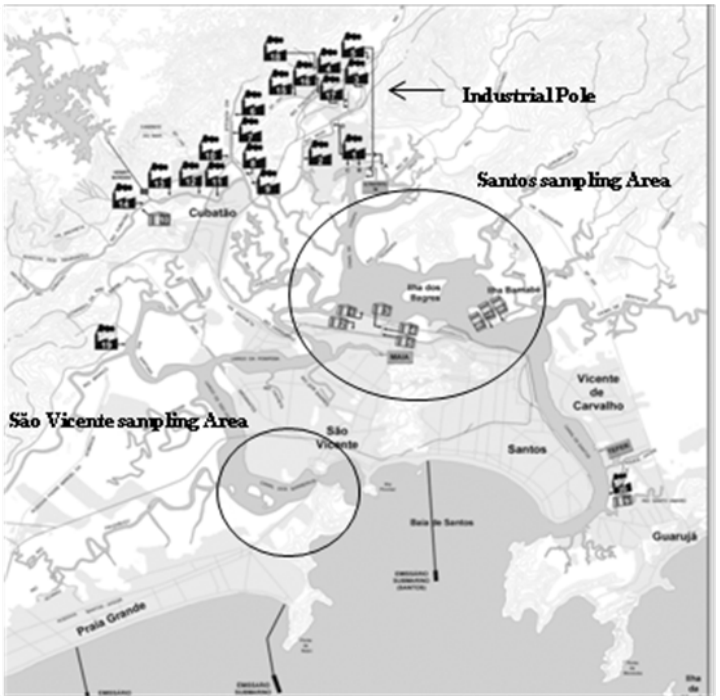

Figure 9. Sources of pollution from Santos and São Vicente estuarine system identifying the industrial pole, and the two fish sampling areas.

Reference Site - Cananéia estuary

The reference site used in this study was the Cananéia estuarine complex. This region is located on the southern coast of the São Paulo state, (between latitude 24^{o}40´00´´ and 25^{o}05´00´´S and longitudes 47^{o}25´00´´ and 48^{o}00´00´´W) (fig. 10). The Cananéia estuary has the highest number of environmental conservation units, sustainable use units and specially protected areas São Paulo state in Brazil. It is also part of the Biosphere Reserve (UNESCO) and has a large permanent preserved Atlantic Forest and mangrove area (Lamparelli et al. 2007).

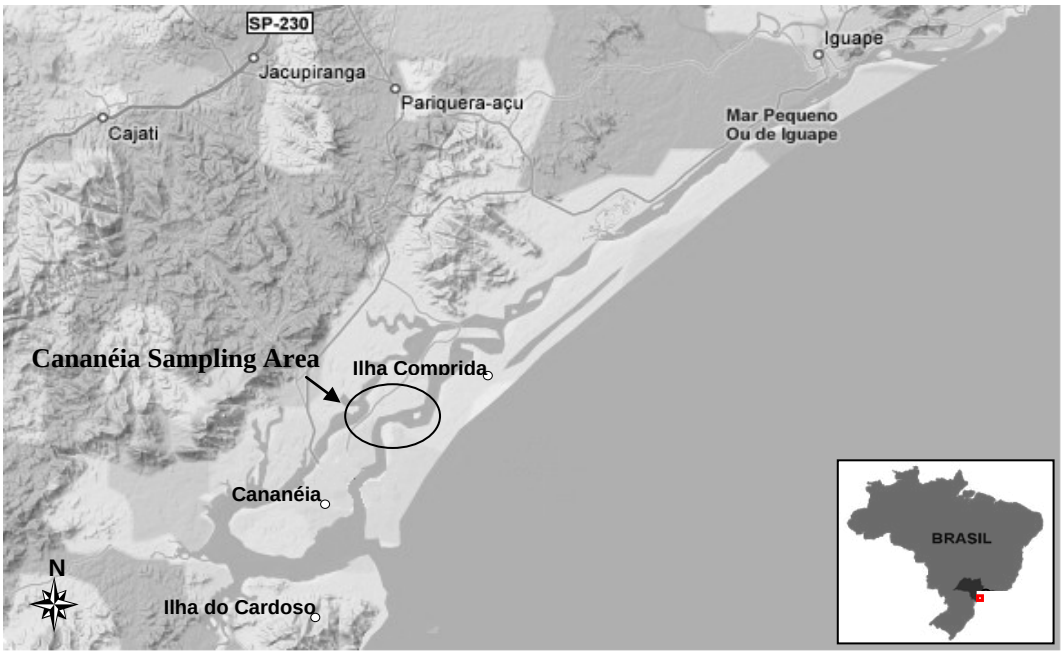

Figure 10. The reference site of Cananéia estuarine complex, where it is identified the fish sampling area.

Sampling

The first fish sampling took place in the winter 2008 and fish brain and muscle were sampled. During the summer of 2009, tissues sampled from collected fish were the liver, brain and muscle. Fish were collected on board of the R/B Albacora ship, using a bottom Otter Trawl (1.600 mesh wall and 1.200 mesh cod end), 11m length and, set at 8.8m depth. Specimens of *C. spixii* were collected in the Cananéia estuarine complex (n = 15) and in Santos (n = 15) and São Vicente (n=15). Collected fish were transported to the laboratory in a thermal box with water and aeration. The identification followed the Figueiredo and Menezes (1978) descriptions. In the laboratory, total length and weight of each fish had been measured. After external measurements, 0.1 g of liver, brain and muscle samples were immediately extracted. The samples had been weighed, stored in criotubes and freeze immediately in liquid nitrogen. Samples were maintained in -80^{o}C until analysis.

Table 1. Mean values of total length and weight from fish sampled from Cananéia, Santos and São Vicente during winter and summer.

Winter	**Length (mm)**	**Weight (g)**
Cananéia	177.86	50.33
Santos	190.66	60.31
São Vicente	194.66	73.90
Summer	**Length (mm)**	**Weight (g)**
Cananéia	182	55.56
Santos	194.13	56.75
São Vicente	177	52.54

Enzymatic Assay

Liver samples were homogenized (1:11) in 0.1 M K-phosphate buffer (pH 7.4) using an Ystral GmbH Dottingen homogeniser. The homogenised sample was divided in two parts, one for LPO analysis and the other part to EROD, CAT, GST and protein determination. For the LPO, 4 µL of BHT methanol was added to each sample and freeze to -80^{o}C for further analysis. The other part of the homogenised sample was centrifuged for 20 minutes at 10000g (4^{o}C)(Eppendorf Centrifuge 5810R). The supernatant was separated in different eppendorfs for EROD, CAT, GST and protein determination. Samples were stored in -80^{o}C for further analysis.

LPO activity in liver was determined by the measurement of thiobarbituric acid reactive substances (TBARS) using a spectrophotometer (Jenway 6405 UV/VIS), according to Ohkawa (1979) and Bird and Draper (1984) methodologies, adapted by Filho et al. (2001) and Torres et al. (2002). The LPO level was expressed in nmol of TBARS/g wt.

Liver microsomes were obtained according to Gravato and Santos (2003) and EROD activity was measured by fixed wavelength fluorescence detection using a Jasco FP-6200 Spectrofluorometer as described by Burke and Mayer (1974). The enzymatic activity was expressed in pmol/min/mg of protein.

CAT activity was determined according to Clairborne (1985) and comprises the consumption of H_2O_2 by spectrophotometry at 240 nm (Jenway 6405 UV/VIS) .

The enzymatic activity was expressed in nmol of substrate hydrolysed/min/mg of protein.

GST activity was determined using a recycling reaction between GSH with 1-chloro-2,4-dinitrobenzene (DTNB). The kinetic activity was read by spectrophotometry at 340 nm (Jenway 6405 UV/VIS) (Guimarães et al. 2009).

Fish brain was homogenised (Ystral GmbH Dottingen homogeniser) in 1 ml of ice-cold phosphate buffer (0.1 M, pH = 7.2), centrifuged for 3 min at 6.000 rpm and 4^{o}C (Eppendorf Centrifuge 5810R). Supernatant was separated and used for ChE and protein determinations. ChE activity was determined by the Ellman method (Ellman et al. 1961), adapted to microplate (Guilhermino et al. 1996) using 0.50 ml of brain homogenate, in a Bio Tek Power Wave 340 microplate reader, at 412nm, and expressed in nmol of substrate hydrolysed/min/mg of protein.

Samples for ChE and LDH activity determinations were prepared by homogenisation of 0.1g of muscle tissue in ice-cold Tris/NaCl phosphate buffer, followed by centrifugation at 6000 rpm for 3 min.(Eppendorf Centrifuge 5810R) at 4^{o}C. The supernatant was then collected and used for enzymatic analysis. ChE activity measurement followed the same methodology described before for brain ChE activity.

LDH activity was determined according to the method of Vassault (1983) adapted to microplate (Diamantino et al. 2001), by measuring the amount of pyruvate consumed, through the continuous monitoring of the decrease in optic density due to NADH oxidation at 340nm (Bio Tek Power Wave 340 microplate reader). The enzymatic activity was expressed in µmol of substrate hydrolysed/min/mg protein.

Protein determinations

Protein concentration in samples was determined in triplicate by the Bradford method (1976), adapted to microplate as indicated in Guilhermino et al. (1996), at 600nm and using the bovine c-globulins as protein standard (Bio Tek Power Wave 340 microplate). Enzymatic activities were expressed as a function of the protein concentration in the samples.

Statistics

One-way analysis of variance (ANOVA) using the Sigma Stat statistical package (SPSS, 1995) was used to evaluate statistical differences between different sampling sites. Whenever significant differences were found, the post-hoc multiple comparison Dunn's method or Tukey test were performed (α=0,05) (Zar 1996). In the Results section, graphs will only elucidate on the statistical results for the One Way ANOVA.

In EROD results, the normality test failed and the statistics was made by Dunn´s method.

A Two-way ANOVA analysis was used to evaluate differences and interactions between enzymatic activities in the three sampling points and season (winter and summer) in brain (ChE) and muscle (ChE, LDH).

Results and Discussion

Santos and São Vicente estuarine system is considered as a highly disturbed and polluted environment. The intense industrial and portuary activities and the irregular occupation of the estuary margins have been induced environmental perturbations in the area (Sousa et al. 2008).

EROD

The EROD activity is one of the best-known and most employed biomarker in ecotoxicology and has been applied as a fish biomarker for several species in many different aquatic environments, but only a few are find with tropical fish species (Al-Arabi & Goksøyr 2002, Cajaraville et al. 2000, Rotchell et al. 1999). EROD activity has not been previously described for any fish species in the Santos and São Vicente estuarine system.

Our results show severe differences (Dunn´s test, P<0,050) between EROD activities from the reference site and Santos and São Vicente estuary (fig.11). The induction of EROD has been described as related to the fish exposure to PAHs, PCBs and related components (Cheikyula et al. 2008, Murphy & Gooch 1997, Rotchell et al. 1999).

The main route of contamination by PAHs into the aquatic environments involve industrial wastes, atmospheric pollution, runoff from adjacent areas, domestic discharges and burn of fossil fuel. The 7-ethoxyresorufin O-deethylase (EROD) enzyme activity at the hepatic biotransformation in liver tissue is specifically induced by PAHs. This makes the EROD activity a good indicator of PAHs contamination (Hartl 2002, Payne & Penrose 1975, Stegeman et al. 1992, Tuvikene 1995). This can explain the significant difference between Cananéia and Santos and São Vicente. Cananéia is a reference site with low contamination levels and, on the other hand and according to the annual monitoring report, published since 2000 by Lamparelli at al. (2007), and the sediment quality evaluation by Abessa (2002), the Santos and São Vicente estuarine system, mainly the Port of Santos channel presents high concentrations of PAHs such as benzo(a)anthracene, benzo(a)pyrene, chrysene, fenantrene, fluoranthene and

pyrene in sediments which can explain the higher activity of EROD in these sites for *C. spixii*, a estuarine bottom catfish species (Katsumiti et al. 2008).

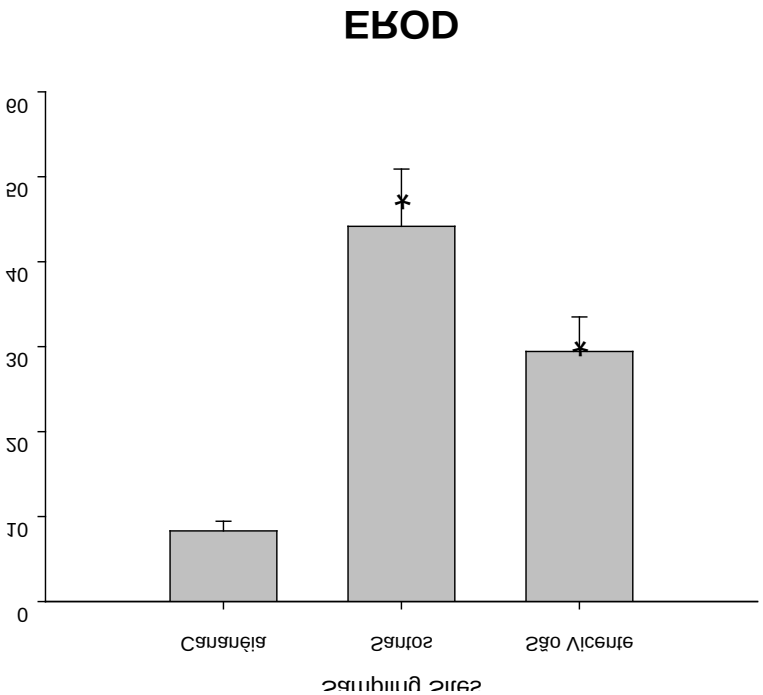

Figure 11. EROD activity obtained in fish liver collected from Cananéia, the reference site, and Santos and São Vicente. The asterisk (*) indicate significantly differences between Santos and São Vicente and the Cananéia (Dunn's Method, P<0,050).

Another fact that explain higher EROD activity in Santos and São Vicente estuarine system is that the PAHs are extremely toxic to aquatic organisms at concentrations around 0.2-10 ppm (Tuvikene 1995). According to Lamparelli at al. (2007), levels of PAHs exceeds that concentration. Examples of PAHs with higher concentrations in sediments from Santos estuary (tab. 1) include benzo(a) anthracene (146 ppm), benzo(a)pyrene (263 ppm), chrysene (175 ppm), fenantrene (112 ppm), fluoranthene (217 ppm), pyrene (310 ppm). Lamparelli et al. (2001) have also concluded in another report that these PAH levels are much higher than levels found in other polluted sites around the world.

Table 2. PAHs concentrations found in sediments from Santos channel according to the Lamparelli et al. (2007) annual report (3nd column) and PAHs concentration limits in sediments from the CCME - Canadian Council of Ministers of the Environment, 2002.

October 2007 Polycyclic Aromatic Hydrocarbons – PAH´s	Concentration from CCME, 2002 (ppm)		Concentration (ppm)
	Limiar effect	Severe effect	
Benzo(a)anthracene	74.8	693	**146**
Benzo(a)pyrene	88.8	763	**263**
Chrysene	108	846	**175**
Fenantrene	86.7	544	**112**
Fluoranthene	113	1494	**217**
Pyrene	153	1398	**310**

Arinç et al. (2000) studied cytochrome P4501A induction in fish liver and found significant relations between contaminated sediments with PAHs from one of the most polluted areas of Turkey and CYP1A induction, as it found in this work.

This results corroborates with the results obtained in the studies from Murphy & Gooch (1997) which evaluated EROD activity in bottom catfishes from an urban estuary and found high significant differences between control, exposure of fishes to benzo-a-pyrene and fishes from an urban estuary with high concentrations of PAHs. Jenkins (2004) also found significant differences in EROD activity from fishes between a contaminated site and a reference site from the

Calcasieu Estuary, Louisiana (USA), where contamination with PAHs and heavy metals were also detected.

Beyer et al. (1996) also studied biomarkers responses in fishes exposed to polluted sediments from Norway and found significant correlation between PAHs and the increase of hepatic EROD activity similarly to the result obtained in this work.

GST

Liver GST activity is related to the biotransformation phase II of several pollutants and the inductions of GST activity have been extensively used as environmental biomarker. GST has been considered the most important phase II enzymes (Monteiro et al. 2005, Stegeman et al. 1992).

Results from this work showed significant differences (Tukey test, P<0,050) between Cananéia, the reference site, and São Vicente (fig. 12). It can be explained by the high concentration of PAHs (tab. 1) and heavy metals (tab. 2) founded by the evaluation of sediment contamination of Santos estuary made by Lamparelli et al. (2007) and Abessa (2002).

Table 3. Heavy metals concentration in sediments from Santos according to Lamparelli et al. (2007) annual report (3nd column) and heavy metal concentration limits in sediments from the CCME - Canadian Council of Ministers of the Environment, 2002.

October 2007 Heavy Metals	CCME, 2002 (ppm)		Concentration (ppm)
	Limiar effect	Severe effect	
Chrome	52.3	160	**57.1**
Mercury	0.13	0.7	**0.49**
Zinc	124	271	**154**

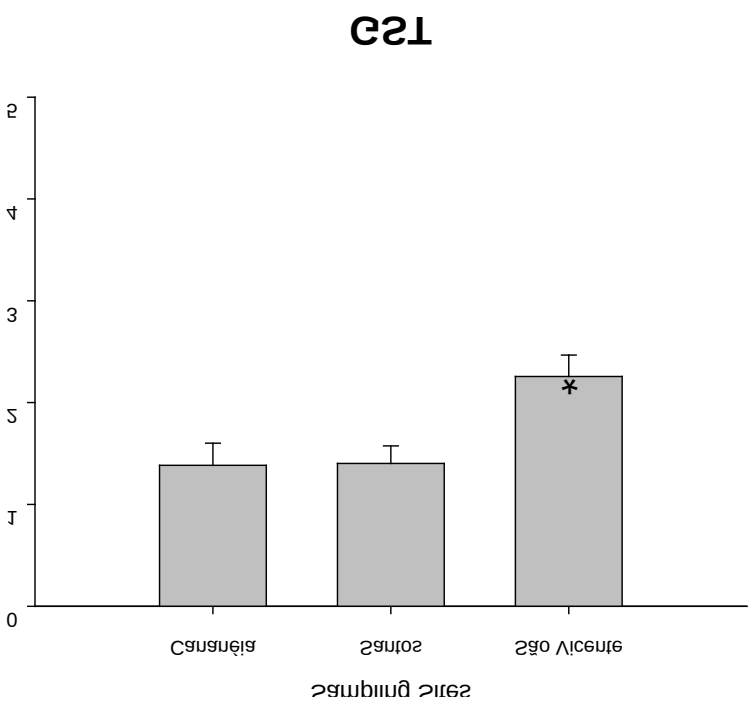

Figure 12. GST activity obtained in fish liver collected from Cananéia, the reference site, and Santos and São Vicente. The asterisk (*) indicate significantly differences between Santos and São Vicente and the Cananéia (Tukey Test, P<0,050).

The higher GST activity in São Vicente corroborates with studies from Monteiro et al. (2007) which studied the impact of chemical exposure on a Portuguese estuarine fish and detected high GST induction according to heavy metals contamination and Farombi et al. (2007) which studied biomarkers of oxidative stress and heavy metal levels in an African catfish and suggested an increase in the GST activity according to an adaptive and protective role against oxidative stress produced by heavy metals.

The similar GST activity founded at Santos when compared to Cananéia can be explained by studies from Yu-qiong et al. (2007) which evaluated antioxidant responses to benzo[a]pyrene and stated that the decline or loss of response in enzymes is related to the inability to adequately respond to contaminants after long periods of exposure.

Another explanation for that low GST activity in Santos site can be explained also by Monteiro et al. (2005) which explain that the enzymatic activity can be inhibited by exposure to contaminants such as metals, PAHs and

pesticides. This contaminants were accumulated in higher concentrations in sediments from Santos site according to the annual monitoring report produced by Lamparelli (2007).

At least, Monteiro et al. (2007) explained that different pollutants can affect enzymes and produce opposite effects, and the general balance may be absence of effect on entire activity.

CAT

The increase in the CAT activity is usually detected in organisms exposed to environmental pollutants because CAT system acts on the first metabolic defence against oxidative stress (Farombi et al. 2007).

In this work, none significant difference has been found between different sampling sites (fig. 13).

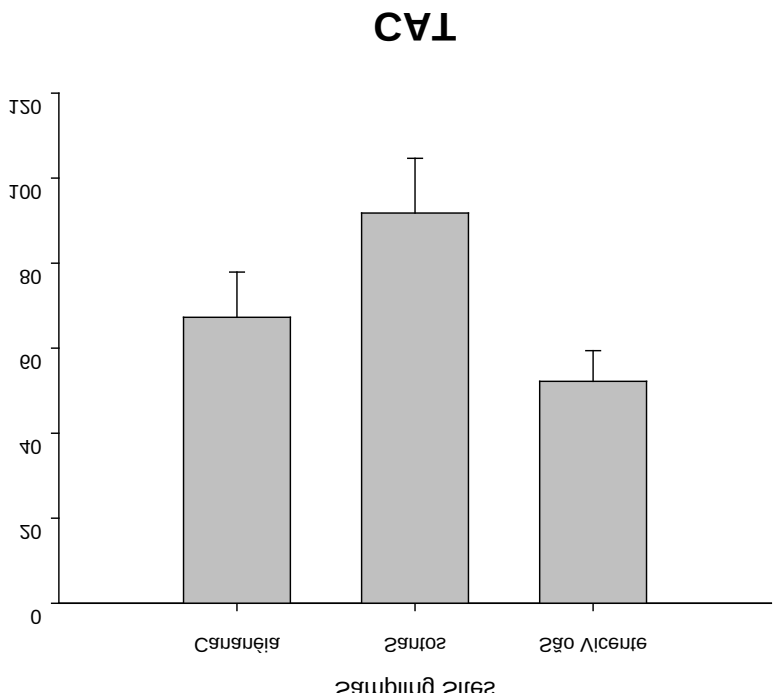

Figure 13. CAT activity obtained in fish liver collected from Cananéia, the reference site, and Santos and São Vicente.

Oost et al. (2003) stated that the increase in hepatic CAT activity was only noticed in fish exposed to sediments contaminated with PAHs, mainly in field studies.

The higher CAT activity found in this work when compared with the chemical analysis from the annual monitored report in Santos sediments corroborate with Oost et al. (2003). According to the report (Lamparelli et al. 2007), higher concentrations of PAHs are found in Santos sediments and it can possibly be the reason for an increase in CAT activity in fishes from Santos site.

Metwally & Fouad (2008) studied the biochemical changes induced by heavy metal pollution in marine fishes and found relation between CAT activity in liver tissue and bioaccumulation of heavy metals in fish. The higher CAT activity in Santos can corroborate with Metwally & Fouad (2008) studies and can be related to the higher heavy metal levels found in sediments from Santos by the annual report study (Lamparelli et al. 2007).

LPO

Lipid peroxidation has been used successfully as a measure of oxidative stress induction promoted by environmental contaminants, especially by heavy metals (Valavanidis et al. 2006).

In this work, the LPO activity did not show significant differences between the different studied sites (fig. 14).

Valavanidis et al. (2006) studied oxidative stress biomarkers in aquatic organisms and found that high concentrations of Zn significantly increase lipid peroxidation and protein content in fish tissues.

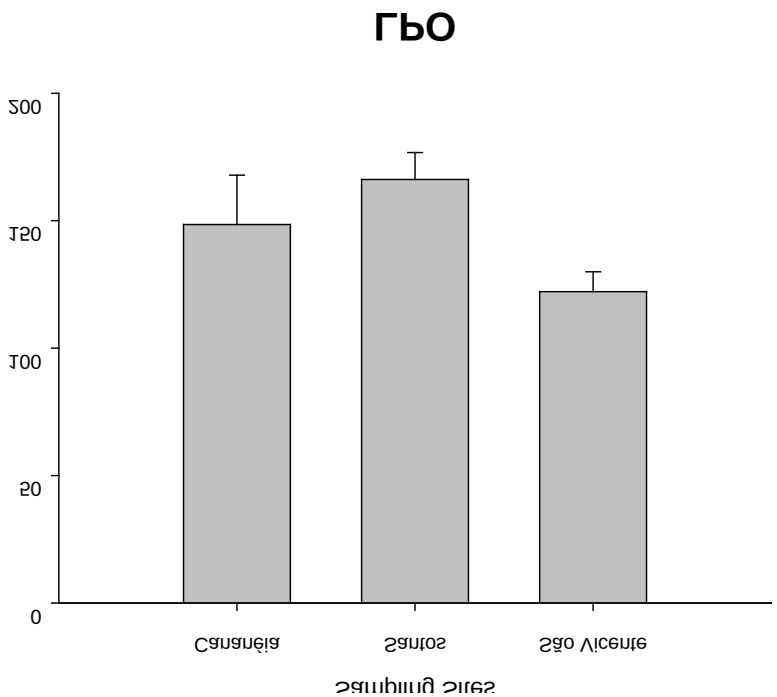

Figure 14. LPO activity obtained in fish liver collected from Cananéia, the reference site, and Santos and São Vicente.

Although reduced glutathione (GSH) analysis was not covered in this work, Yu-qiong et al. (2007) stated that the protective effect of GSH is due to the ability of blocking the LPO activity which can explain the absence of significant differences in LPO activity between the three sites studied.

Stegeman et al. (1992) presents in their work that catfishes exposed to sediments contaminated by PAHs could increase LPO induction in various fish tissues, which can be represented in this work at Santos site, the highest LPO levels at the most contaminated site by PAH (Lamparelli et al. 2007) in this study.

ChE

Acetylcholinesterase ChE inhibition is linked directly with the mechanism of toxic action of organophosphorus, pesticides and contaminants like mercury (Cajaraville et al. 2000).

According to Lamparelli et al. (2007) higher levels of organophosphate and mercury (tab. 2) have been found in Santos sediments. This can explain the

significant difference (Tukey, P<0,050) found between Santos and other sampling sites in winter (fig. 15 A) and small ChE inhibition in brain tissues from São Vicente and Santos at summer (fig. 15 B).

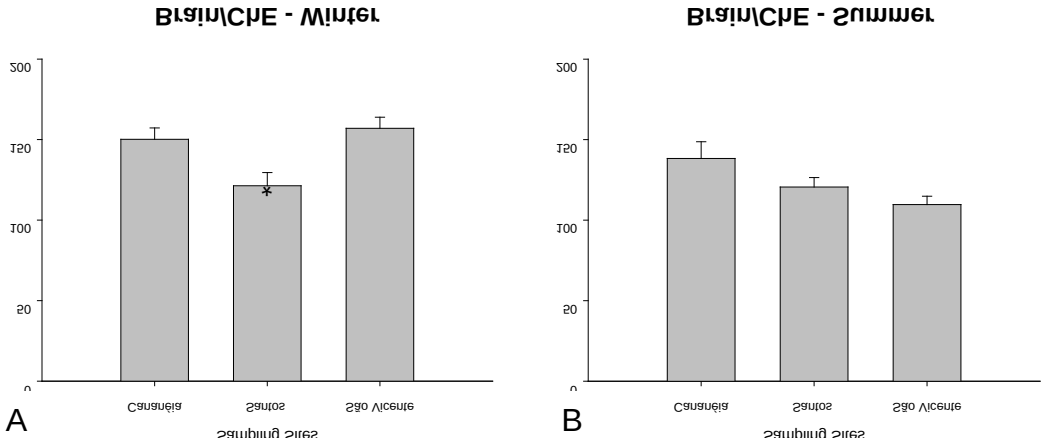

Figure 15. ChE activity obtained in fish liver collected from Cananéia, the reference site, and Santos and São Vicente in winter (A) and summer (B). The asterisk (*) indicate significantly differences in Santos at winter (Tukey Test, P<0,050).

Linde-Aria et al. (2008) used ChE as part of a multiple biomarker analysis to evaluate the impact of pollution in a Brazilian river and found similar results when compared to this work (fig. 15 A, B), with a ChE inhibition pattern between polluted sites and the reference site.

Katsumiti et al. (2008) studied an oil spill occurred at Paranagua Bay, Brazil, and evaluate effects in ChE activity from fish muscles (*C. Spixii*). After the exposure to oil spill the ChE activity from the contaminated site presents inhibition in relation to the reference site, similar to what have been found in this work (fig. 15 A) with muscle analysis from winter samples.

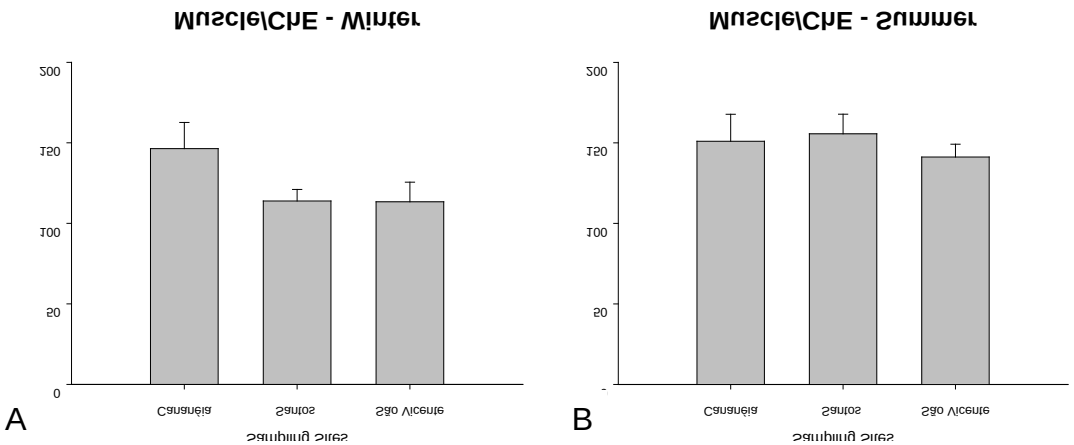

Figure 16. ChE activity obtained in fish liver collected from Cananéia, the reference site, and Santos and São Vicente in winter (A) and summer (B).

The possible explanation for the small differences found between the different sites in different season could be the fact that ChE activity is altered mainly by pesticides, a contaminant characteristic from agricultural areas. Santos and São Vicente are mainly influenced by industry effluents and contaminations by heavy metals and PAHs (Abessa 2002, Cajaraville et al. 2000).

Small differences in the ChE activity observed in winter may be promoted by higher concentration of and metals found at Santos and São Vicente (Abessa 2002, Cajaraville et al. 2000, Lamparelli et al. 2007).

In the between ChE activities, interactions have been found between season and sampling points in brain (Two way ANOVA, $F_{2, 80}=5,40$, $P<0,050$).

Differences found in brain ChE activity shows a higher ChE inhibition in Santos and São Vicente in summer in relation to the same sites in winter, despite significant difference can be found in Santos in relation to Cananéia and São Vicente in winter. The higher summer ChE inhibition can be explained by the increase of tourism influences (1,5 million people) in summer season which promote higher contribution of domestic sewage to the estuary. The summer season correspond to the rainy season which can intensify the contribution of runoff from adjacent areas to the estuary (Sousa et al. 2008).

LDH

The LDH activity has also been used to evaluate toxic effects and assess alteration in metabolism of vertebrate and invertebrate organisms (Greco et al. 2007).

In this work significant differences (Tukey, P<0,050) were found between Santos and the different sampling sites in winter (fig. 17A). None differences in Santos and São Vicente were detected in relation to the reference site in summer in relation to Cananéia (fig. 17B).

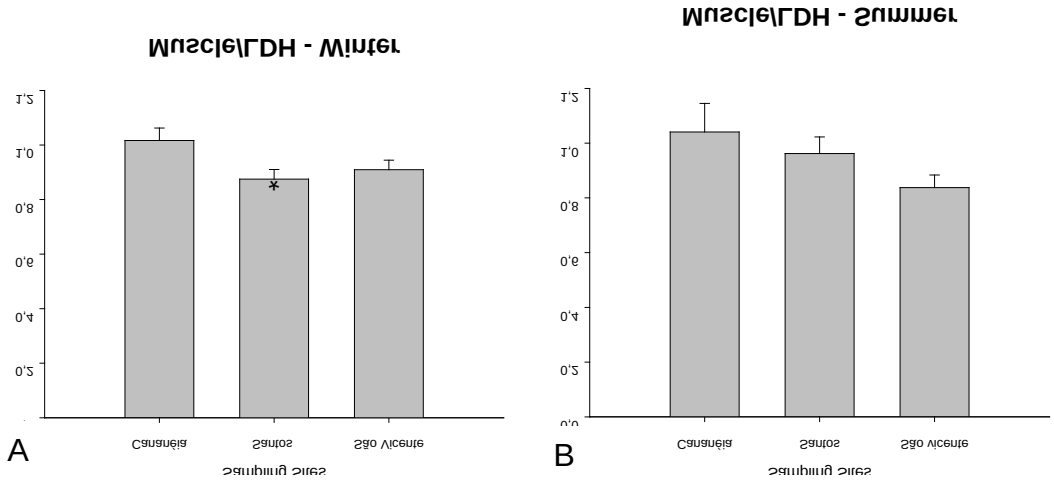

Figure 17. LDH activity obtained in fish liver collected from Cananéia, the reference site, and Santos and São Vicente in winter (A) and summer (B). The asterisk (*) indicate significantly differences between Santos and São Vicente and the Cananéia (Tukey Test, P<0,050)

The differences on LDH activity in relation to Cananéia can be occurred due to the higher concentrations of heavy metals, PAHs and by the intense portuary operations from fertilizers shipments. The operations of loading ships promote the discharge of part of the load to the water port channel as it occurs with fertilizers shipments. Additionally to fertilizer shipments, some fertilizer industries are presents at the Santos and São Vicente estuary and possibly promote negative influences to the ecosystem by the discharge of effluents (Nishigima et al. 2001).

Yadav et al. (2007) studied the induction of fertilizers industry effluent in biochemical changes and stated significant alterations in LDH activity.

No interaction in seasonality and LDH activity were observed between Santos, São Vicente and Cananéia.

The results from this study demonstrate that a biomarker battery is useful to evaluate different detoxification phases and to define the exposure and effects of anthropogenic inputs among impacted and reference sites. The native fish species *C. spixii* demonstrated to be a suitable sentinel species to assess the effects of pollutions in this aquatic system.

The high activity observed at the first phase of liver detoxification through EROD analysis and in the second phase by GST activity can indicate that xenobiotic compounds can be biotransformed and eliminated at the phase I and II of the detoxification process, which can dimish differences in the other enzymatic analysis.

Chapter III - Conclusions

The results from this study demonstrate that a biomarker battery is useful to evaluate different detoxification phases and to define the exposure and effects of anthropogenic inputs among impacted and reference sites. The native fish species *C. spixii* demonstrated to be a suitable sentinel species to assess the effects of pollutions in Santos and São Vicente estuarine system.

The first phase in liver detoxification through EROD activity shows to respond to the contaminant levels in Santos and São Vicente. The high PAHs concentrations found in Santos and São Vicente estuary sediment presented in the annual environmental monitoring program corroborate with this findings as the first phase of detoxification is known to be activated mainly by PAHs.

The second phase in liver detoxification, represented here by the GST enzymatic activity, presents significant differences at São Vicente in relation to Cananéia and Santos. The GST activity can be inhibited in case of severe environmental contamination by metals, PAHs and pesticides, contaminants known as present in sediments of Santos estuary or by long term period of exposure to PAHs.

ChE activity presents small differences between samples site and seasonality. At winter, Santos presents higher inhibition maybe because of the high concentration of mercury found in Santos sediments. In summer, none significant difference has been observed.

In this study responses from CAT and LPO activities were expected to be in accordance to contaminants present in the studied sampling area. But that did not occur, at least significantly.

LDH activity is significant different in summer at Santos site and presents small inhibition in Santos and São Vicente in winter. These differences can be related to fertilizers shipment at the portuary area.

The high activity observed at the first phase of liver detoxification through EROD analysis and in the second phase by GST activity can indicate that xenobiotic compounds can be biotransformed and eliminated at the phase I and II of the detoxification process, which can explain the smaller differences at the others enzymatic analysis.

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Annex

Energy budget in *Daphnia magna* exposed to natural stressors

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Abstract

Background, aim, and scope Climate changes are nowadays an important issue of concern and it is expected that in the near future there will be an increase of frequency of extreme environmental conditions. These changes are expected to originate new sources of stress, therefore the exposure of organisms to natural stressors is receiving greater importance in risk assessment. Organisms tend to avoid extremely environmental conditions, looking for an intermediate and adjusted range. This work aimed to evaluate the effects of natural stressors on the energetic reserves of *Daphnia magna* by quantifying lipids, proteins and sugars. The tested hypothesis is that during periods of stress which lead to a higher energy demand, these energy reserves are mobilized.

Materials and methods To test our hypothesis daphnids were exposed to different temperatures ranges (16°C; 18°C; 22°C; 24°C and 26°C), food levels (2, 1.5, 1, 0.5, 0 and 4, 4.5, 5, 5.5, 6 x10^5 cells/ml *Pseudokirchneriella subcapitata*) and oxygen depletion (2 to 6 mg DO/L), and their energy reserves quantified Protein, lipid and sugar contents where compared between daphnids exposed to the specific stress conditions and those in the control.

Results and Discussion Significant changes in energy reserves content after a 96h exposure were observed in temperatures 16ºC, 22ºC, 24ºC and 26ºC. In the exposure to different food levels, daphnids showed significant differences on their energetic reserves when exposed to higher or lower levels of algae when compared to the control. Oxygen depletion did not affect significantly their energy budget.

Conclusions The results from this work demonstrate that the environmental alterations related mainly to temperature variations and food availability produced changes in *Daphnia magna* energetic reserves. These changes can be transposed to the population level as they are a result of changes in the metabolic rate and physiological processes that are related to growth and maturation.

Key-words natural stressors; risk assessment; energy budget; Daphnia magna

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1 Background, aim, and scope

Climate changes are nowadays an important issue of concern and it is expected that in the near future there will be an increase of the frequency of extreme environmental conditions. These changes are expected to originate new sources of stress and therefore the exposure of organisms to natural stressors is receiving a greater importance in risk assessment (De Coen & Janssen 1997, IPCC 2007).

Organisms tend to avoid extremely environmental conditions (e.g. higher or lower temperatures, absence or excess of food and oxygen) looking for optimum conditions, like intermediate and adjusted ranges. When they are unable to avoid deleterious conditions, they tend to adapt by altering their physiological processes in order to decrease their energy consumption. Studies to evaluate the preference of organisms to thermal changes have been carried out and daphnids, among other crustaceans, are one of the most used test-organism (Lagerspetz 2000). Daphnids are ectothermal and eurythermic organisms and their body temperature changes according to the external (environmental) temperature, causing alterations in physiological and biochemical processes (Tsui & Wang 2004). Changes on environmental temperature are known to promote the development of mechanisms, i.e, adaptations, on feeding activity, delay in maturation and egg development, to warrant the survival of future populations (Mourelatos & Lacroix 1990, Overgaard et al. 2008, Pieters & Liess 2006, Rinke & Petzoldt 2003, Rinke & Vijverberg 2005, Simcic & Brancelj 2001).

Another important factor which influences the population survival success is the variation of food type and availability. Studies made with daphnids exposed to different food concentrations suggested alterations on eggs production, individual growth and consequently on population levels. In absence of food or food quality, organisms that suffer from starvation may sacrifice their individual growth and prejudice the maturation and consequently their reproduction success in order to survive (Goulden & Hornig 1980, Nogueira et al. 2004, Pieters & Liess 2006, Rinke & Petzoldt 2003, Rinke & Vijverberg 2005).

Rinke and Petzoldt (2003) showed that high food levels increases daphnids body length. On the other hand, high food levels in aquatic environments might lead to an increase of animals and indirectly increase oxygen consumption, which leads to another natural stressor: oxygen depletion. Hypoxic conditions can cause physiological responses like the increase of heart and appendage beating rates, increasing reserve consumption and consequently prejudice organisms' growth, reproduction and population survival (Hanazato & Dodson 1995, Seidl et al. 2005).

Some studies testing the effects of natural stressors have been carried out at the population level, but molecular biomarkers can be used to early predict the effect of stressors in individual metabolism, and transposing to effects on the population and later to the ecosystem (Den Besten 1998). Although energy reserves are considered as long term exposure biomarkers, they have proved to respond to chemical stressors in daphnids in short term exposures (De Coen et al. 1998, Mayer et al. 1992).

This work aimed to evaluate the effects of natural stressors on the energetic reserves of *Daphnia magna* by quantifying lipids, proteins and sugars. We have raise up the hypothesis that during periods of stress which lead to a higher energy demand, these energy reserves are mobilized. To test our hypothesis daphnids were exposed to different temperatures ranges, food levels and oxygen depletion, and their energy reserves quantified.

The results of this research will be crucial for a better understanding of the results from combined exposures of *Daphnia magna* to natural stressors and chemical compounds.

2 Materials and methods

2.1 Daphnia magna culture

The cladoceran *Daphnia magna* Straus, clone K6 (originally from Antwerp, Belgium) is being cultured in our laboratory for up to 3 years. Cultures were maintained in three 20L glass aquarium with 100L of ASTM hard water (ASTM 1980), renewed and fed three times a week with *Pseudokirchneriella subcapitata* (Korshikov) Hindak (3 x 10^5 cells/ml) and with an organic extract (Baird et al. 1989). About 250 daphnids per aquarium (10L capacity) were kept in a 16:8h light:dark cycle and temperature of 20±1^{o}C (Ferreira et al. 2007). Neonates from the third to fifth broods were used in tests and neonates from sixth broods were used to re-start new cultures.
A potassium dichromate ($K_2Cr_2O_7$) reference test was carried out before the beginning of all test exposure to evaluate daphnids' sensitivity.

2.2 Natural Stressors

The natural stressors tested were food quality and quantity, different temperatures and dissolved oxygen concentrations (DO). Tests run for 96 hours. In all experiments control conditions were carried out at a temperature of 20°C, photoperiod of 16:8 (light:dark), ASTM hard water with 9mg/L DO and daphnids were feed with 3x10^5 cells/ml *Pseudokirchneriella subcapitata* and organic extract (ASTM 1980, Baird et al. 1989). Test exposures were performed in a 1L glass flask with 800mL of ASTM hard water, renewed every other day. Four flasks containing 45 daphnids each were used for each test treatment and control.
Two types of food were used for the food type test. One was carried out only with the green algae *P. subcapitata*, and the other with a mixture of *P. subcapitata* with 6 ml of organic extract per liter (as in the culture conditions).
For the food concentration testing, daphnids were exposed to different concentrations of the green algae *P. subcapitata* and a constant organic extract (as used in culture conditions), and experiments were divided in two sets: high food concentration and low food concentrations. The higher concentrations tested were 4, 4.5, 5, 5.5 and 6 (x10^5 cells/ml) and the lower concentrations were 2, 1.5, 1, 0.5 and 0 (x10^5 cells/ml). In each set a control beaker, with daphnids fed with 3x10^5 cells/ml *P. subcapitata*, was used.
To evaluate the effects of different temperatures in energy reserves, daphnids were exposed to: 16°C; 18°C; 22°C; 24°C and 26°C. Tests were carried out in rooms or chambers with controlled air temperature (Biotronette Chamber, Lab Line Instruments); 20ºC was the control temperature.
For the dissolved oxygen tests, oxygen concentrations ranged from 2 to 6 mg DO/L, as sub-lethal levels, based on the study of Ferreira et al. (2008). To achieve these oxygen levels a controlled atmosphere chamber (model 855-AC, Plaslabs, USA) was used to assure different ranges of oxygen in the ASTM medium; DO

was measured inside the chamber using an oxygen meter (WTW 330i, Germany). 100ml gastight glasses (Schott®) were used to avoid the loss of oxygen during the test period. Food was added after DO was settled. Test medium was renewed inside the controlled atmosphere chamber, after 48 hours of exposure. The concentration of 9mg of oxygen /L was assumed as the control concentration.
The exposure to each stressor lasted for 96hs. Daphnids were separated in groups of 30 in eppendorfs and instantaneously frozen in liquid nitrogen to use for the energy budget dosage.

2.3 Energy reserves

Total lipids were extracted following the methodology described by De Coen and Jansen (1997). Daphnids were homogenized in 450µl distilled water using a plastic pestile. After homogenization, 500µl chloroform (spectrofotometric grade) and 500µl methanol (spectrofotometric grade) were added. After samples centrifugation at 3500 r.p.m. (Eppendorf Centrifuge 5810R) the top phase was removed and 500µl H_2SO_4 were added to the 100 µl lipid extract and heated for 15 min at 200^{o}C. The total lipid content was determined by measuring the absorbance at 370 nm in a microplate reader (Labsystem Multiskan Ex); tripalmitin was used as a standard.
To determine total protein and carbohydrate (sugar) content, daphnids were homogenized using a plastic pestile in 200µl distilled water. After homogenization, 15% trichloroacetic acid (TCA) was added and samples were incubated at –20^{o}C for 10 min. After centrifugation at 3500 r.p.m (Eppendorf Centrifuge 5810R), the formed pellet was washed with 5% TCA and both supernatant fractions were combined and used for the total sugar analysis. The remaining pellet was re-suspended in NaOH and incubated at 60^{o}C for 30 min and then neutralised with HCl. Total protein content was determined using the Bradford's method (Bradford 1976). The absorbance was measured at 590 nm in a microplate reader (Labsystem Multiskan Ex) and the standard used was bovine serum albumin. Total carbohydrate content of the supernatant fraction was quantified by adding 5% phenol and H_2SO_4 to the extract. After 30 min incubation at 20^{o}C, the absorbance was measured using glucose as a standard at 492 nm in a microplate reader (Labsystem Multiskan Ex). Carbohydrate reserves will be hereafter designated as sugar contents.

2.4 Statistical analysis

One-way analysis of variance (ANOVA) using the SigmaStat statistical package (SPSS, 1995) was used to evaluate statistical differences between treatments. Whenever significant differences were found, the post-hoc multiple comparison Dunnett's method or Tukey test were performed (α=0.05) (Zar 1996). When only two groups were compared a t-test was used.

3 Results and Discussion

3.1 Different Temperature

In this study the increase of temperature enhanced daphnids energetic reserves contents (Fig.1). Higher temperatures showed significant differences when compared to the control temperature of 20ºC in both the sugar and protein contents (Dunnet test, $P<0.001$) protein concentration duplicated and sugar concentrations increased five times. Significantly differences were also found between lipid contents of daphnids exposed at 24ºC when compared to control exposures (Dunnet test, $P<0.001$).

The increase of protein content in higher temperatures, as observed for 22^{o}C, 24^{o}C and 26^{o}C (fig. 1B), might be related to the increase on hemoglobin (Hb) concentrations among other proteins as temperature increased, as found by Zeis et al. (2004). Wiggins and Frappell (2002) also found a relationship between different temperatures, metabolic activity and Hb increase. They concluded that the metabolic activity increases in higher temperature to compensate the adequate levels of oxygen, which is altered by higher temperatures. This will lead to an increase on Hb content promoting a better oxygen extraction from the environment.

Below 20ºC, daphnids showed similar contents on lipids and sugar when compared to the control temperature of 20ºC. When exposed to 16ºC the protein fraction was significantly affected, showing a decrease around 41% when compared to the control. Mourelatos and Lacroix (1990) also found effects of different temperature exposures on the feeding activity of cladocerans, with an increase on grazing activities when temperatures were raised from 10 ^{o}C to 20 ^{o}C.

At low temperatures, organisms usually use their energy to maintain their metabolism and at higher temperatures organisms spend less energy and are able to store reserves. Overgaard et al (2007) studied alterations on sugar contents near freezing temperatures in earthworms and detected the rise of sugar content in below zero temperatures, which is different from the results found in this work where lower temperatures promote a decrease in sugar content. Despite different temperature ranges were used in both works due to different life-cycle and life strategies of test organisms used.

Daphnia is usually thermal sensitive to changes of 0.2ºC to 0.5^{o}C, which can probably explain the significantly differences observed even in small variations on temperature of 2^{o}C (Lagerspetz 2000). In addition, *D. magna* has shown to diminish its antennae downward strokes activity at temperatures below 20^{o}C. The decrease of antennae movement can be related to the attempt to maintain their metabolism and decrease reserve loss used for swimming movements, decreasing their feeding rates. Therefore differences in temperature and food consumption are also known to be correlated. The filtering rate increases as temperature increases, and that fact can explain why energy reserves contents raised while temperature exposures were higher (Mourelatos & Lacroix 1990).

Simcic & Brancelj (2001) studied the seasonal dynamics of metabolic activity at the *Daphnia* community in Lake Bled (Slovenia) and concluded that temperature is the most important environmental stressor that affected metabolic activity in *Daphnia* leading to alterations in reproduction and respiration rates.

3.2 Food Concentration

In the food concentration test high food concentrations induced an increase on energy reserves when compared to the control (*fig. 2*). These differences were more pronounced at 5.5 and 6 $x10^5$ cells/ml for proteins and 5 to 6 $x10^5$ cells/ml for sugar's contents.
Rinke and Petzoldt (2003) studied the effects of temperature and food on individual growth of *D. magna* and observed a clear relationship between higher food concentrations and increases of Daphnia body length and maturity. This might explain the higher content of reserves in higher food concentrations evaluated in this work, as daphnids with 96h old had reached already their maturation and some have already eggs on their marsupium (data not shown).
In lower food concentrations (*fig. 3*), energy reserves decreased with the decrease of food supplied. This possibly happens because low concentrations of food make organisms use their own energy reserves to compensate the absence of proper food. Several authors also suggested that the constant filtration activity of Daphnia and digestion of ingested food by biochemical transformation can lead to a higher spent of energy reserves (e.g. De Coen & Janssen 1998). It was not possible to detect protein or sugar contents with the methodology used when daphnids were left with no food.
Low food concentrations can slow growth and delay daphnids maturation. Studies with temperature variations and food concentrations confirm these patterns (Rinke & Vijverberg 2005). In our study test-organisms exposed for 96 hours to low food concentration showed smaller sizes than those from the control conditions (data not shown).
Another explanation for the observed low food concentration results can be related to the findings of De Coen and Janssen (1998). They studied the ingestion and digestive enzyme activity in *Daphnia magna* and suggested an energy saving strategy caused by environmental stress. In this energy saving strategy, organism diminishes metabolism and reduce feeding activity to save the energy available (De Coen & Janssen 1998). This mechanism can make possible sufficient assimilation efficiency and maintain *Daphnia* metabolism despite a decrease in the ingestion rate.

3.3 Dissolved Oxygen depletion

Experiments carried out with different concentrations of dissolved oxygen (*fig.4*) show small but non significant differences in reserve contents after 96 hours of exposure.
Environments with oxygen depletion characteristics might became challenging to freshwater organisms (Wiggins & Frappell 2002). *Daphnia* can live in eutrophic aquatic environments and be exposed to high variable oxygen conditions by adjusting its circulatory system and hemoglobin expression. *Daphnia* exposed to hypoxia shows alterations on their aerobic metabolism, causing alterations in physiological functions and organism growth (Seidl et al. 2005). So it was expected that energy reserves were significantly affected by oxygen depletion. Small variations were observed for protein and sugar contents, although with opposite trends. Oxygen depletions (2 and 3 mg/L) induced a small increase on sugar contents and a small decrease on protein contents.

In the case of hypoxia, *Daphnia* metabolism diminishes energy demand to balance the difficulty in obtaining oxygen from the stressed environment (Seidl et al. 2005). That reduction in energy demand can make daphnids preserve their reserves, using them as least as possible when exposed to inadequate oxygen levels. That maintenance in energy reserves could be seen in figure 4, where only small and non significant differences were observed between dissolved oxygen concentrations.

3.4 Food type

In the food quality experiments, small differences between green algae and green algae plus organic extract (*Fig. 5*) were observed in energy reserves contents, during the 96h of exposure.
This kind of approach is important because the type of food could promote alterations in size and number of offspring, decreasing the chances of population to survive (Pieters & Liess 2006). Although our results do not present significant differences, tests with different food types are very important because in a stressed environment organisms will fed making use of the food available at that moment, which sometimes is not the ideal for the healthy maintenance of the population.
In addition, our results might also be related to the short exposure time; for a better evaluation of different food type effects in the reserves content a longer exposure time is suggested. Mayer et al. (1992) studied physiological and nonspecific biomarkers and concluded that changes in energy reserves are generated by long-term exposure, although the 96 hours short-term tests used here presents good results with significant differences, mainly with different temperature and food concentration. Goulden and Hornig (1980) studied the oscillations and energy reserves in planktonic cladocera and enlightened that Daphnia from lab cultures have the characteristic of using their energy (content) instantly to maintain their metabolic activity. This can explain the rapid effect observed in short-term tests at the energetic content.
All of our results demonstrate that the exposure of *D. magna* to natural stressors can cause changes in their energy budget. Previous studies have shown that energy measurements can be sensitive indicators to evaluate environmental stress (Canli 2005, De Coen & Janssen 1997, De Coen & Janssen 1998, De Coen et al. 2001, Holmstrup et al. 2007). Other studies have used individual energy reserve fractions such as sugars, lipids and protein content as general indicators of stress and the response pattern is stressor dependent (De Coen & Janssen 1997).

4 Conclusions

In a world facing several environmental alterations like climate changes and alterations caused by human activities, the evaluation of natural stressors effects on environmental compartments is receiving increased attention. Results of this work demonstrate that the environmental alterations, related mainly to temperatures variations and food availability, produced changes in *Daphnia magna* energetic reserves, especially on protein and sugar contents. These changes can be transposed to the population levels, as they are a result of changes in metabolism rates and physiological processes that are related to growth and maturation.

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Fig. 1 Effect of a 96h exposure to different temperatures on the lipidic (A), proteic and sugar (B) contents in *Daphnia magna*. The asterisk (*) indicates significantly differences between treatments and the control ($P \leq 0.05$). In graph B, black bars refer to proteins and grey bars to sugar contents.
Fig. 2 Effect of a 96h exposure to different food concentrations (higher levels) on the lipidic (A), proteic and sugar (B) contents in *Daphnia magna*. The asterisk (*) indicate significantly differences between treatments and the control ($P \leq 0.05$). In graph B, black bars refer to proteins and grey bars to sugar contents.

Fig. 3 Effect of a 96h exposure to different low food concentrations on the lipidic (A), protein and sugar (B) contents in *Daphnia magna*. The asterisk (*) indicate significantly differences between treatments and the control ($P \leq 0.05$). In graph B, black bars refer to proteins and grey bars to sugar contents.
Fig. 4 Effects of a 96h exposure to different concentrations of dissolved oxygen on the lipidic (A), protein and sugar (B) contents in *Daphnia magna*. In graph B, black bars refer to proteins and grey bars to sugar contents.
Fig. 5 Effects of a 96h exposure to different food type on the lipidic (A), protein and sugar (B) contents in *Daphnia magna*. In graph B, black bars refer to proteins and grey bars to sugar contents.

Fig. 1

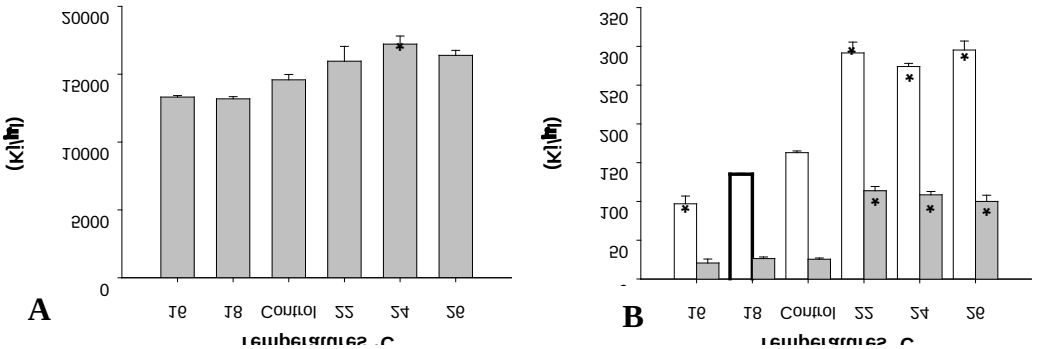


Fig. 2

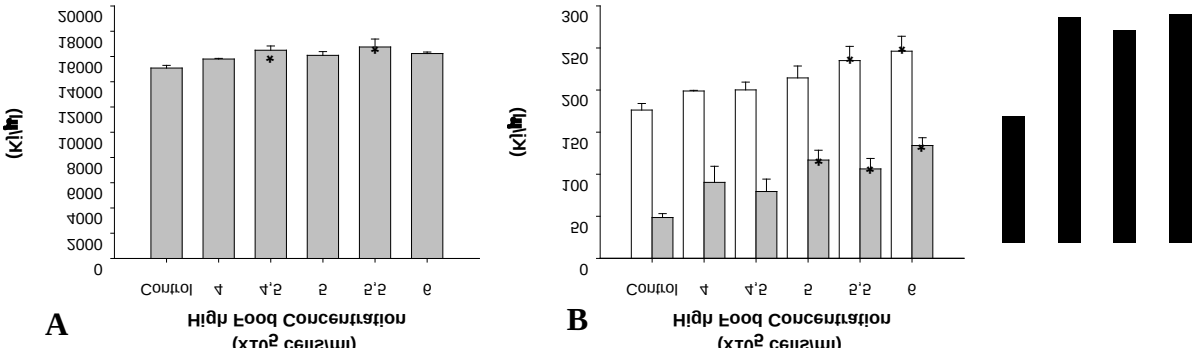
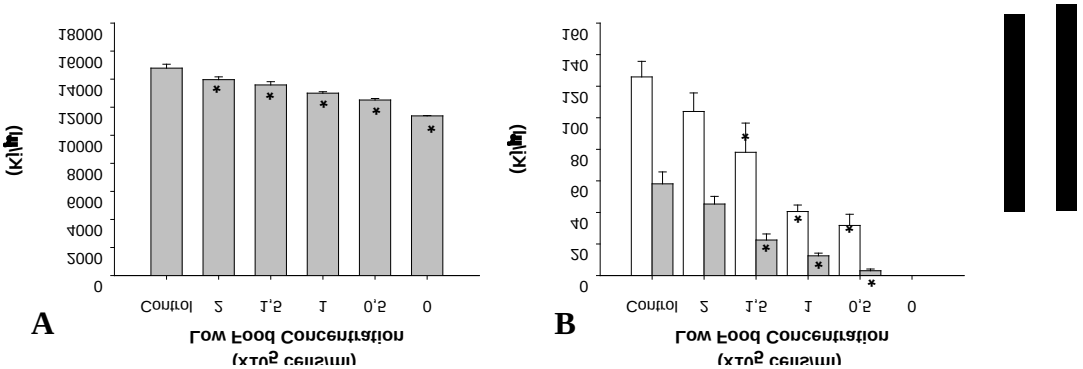


Fig. 3



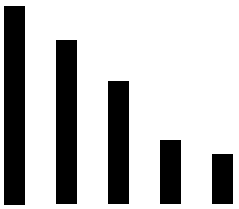


Fig. 4

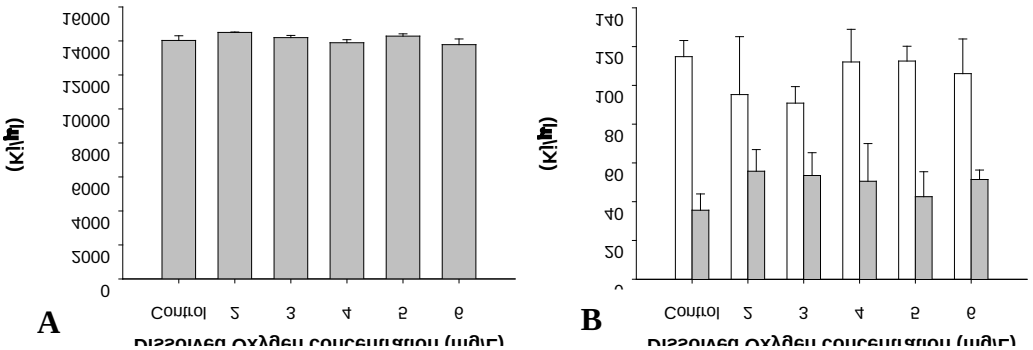


Fig. 5